Specification

Title of the Invention

MEANS FOR STABILIZING HEMOGLOBIN

This application is a 371 of PCT/JP00/0440 filed on 07/03/2000 which claims benefit of priority to Application No. 11/188132 filed in Japan on 07/01/1999.

Field of the Invention

The present invention relates to a method for stabilizing hemoglobin and a composition containing stabilizing hemoglobin.

Background of the Invention

Hemoglobin is a heme-protein-existed in red blood cell, which is constituted with tetramer consisting of 2 pair of polypeptide chains, (each chain is bound to 1 molheme), named as α- and β-chains, and having a molecular weight of 65,000. The hemoglobin is contained in blood in an amount of 16 to 18 g/dL for male, and 14 to 16 g/dL for female, and play an oxygen transporting function by reversibly attaching/detaching oxygen to iron.

Because of maintaining such a rule, a measurement of the hemoglobin is one of the basic measurement items in a clinical laboratory test, and is utilized as a diagnosis of anemia, i.e., iron deficiency anemia, hypoplastic anemia, hemolytic anemia, and etc., in combination with red corpuscle number and hematocric value. Recently, by detecting an extremely small amount of hemoglobin contained in a stool, the measurement, is advantageous in the diagnosis of colon cancer.

On the other hand, a glycated hemoglobin is produced by non-enzyme reaction between hemoglobin and glucose. A measurement of the glycated hemoglobin in blood is reflected with a control of blood sugar concentration for past 3 or 4 weeks, and is effectively available as an indication of blood sugar concentration in long term, which is not affected by daily meals. In the present specification, hereinafter, the term hemoglobin is used, as including the total of hemoglobin and glycated hemoglobin.

At present, the measurement of the hemoglobin in clinical laboratory takes place with HPLC method, immunological method, affinity method, electrophoresis method, isoelectric fraction method, TBA (2-thiobarbituric acid) method, RIA (Radio Immuno Assay) method, Photic acid method, furosine determination method, and the like.

On measuring the hemoglogin above, a substance which may be of standard and control material is required. However, the hemoglogins are unstable, and lose their oxygen binding function, and heme iron is changed from Fe^{2+} to Fe^{3+}, by oxidation, and change in color from clear red to dark brown. Furthermore, hemoglobin is noted to be oxidized to methemoglobin, by exposure thereof to air and by lyophilization.

As a method for protecting such an oxidization, there is a method for which, on a site, where an oxygen is to be bound, two moles of atoms, such as CO, NO, CN, etc., which have almost the same diameter as that of oxygen, are previously bound, or a method for adding sodium azide on lyophilization step. However, these methods have drawbacks, in that the operations are troublesome; one of the methods provides significantly inadequate low amount, or another is impossible in operability. Besides these, a disposal problem regarding sodium azide arose due to poisoness. Further, even though a method for adding a compound containing nitrogen is disclosed in the publication (Laid-open Japanese Patent Publication No.Sho60-35270), an effect for preventing separation of free iron form from hemoglobin is merely disclosed. A method for incorporating glucose and amino acids (Laid-open Japanese

Patent Publication No. Sho 61-1620) discloses a protecting agent for oxidizing hemoglobin to methemoglobin, an object of the method, however, is to stabilize hemoglobin which are used as a substitute blood having a concentration mg/mL level, as hemoglobin. Thus, these methods are not acceptable for stabilizing hemoglobin having a concentration in ng to μg/mL, which is required in clinical laboratory test as a standard or control material. Another method for stabilizing hemoglobin by incorporating amino acid and albumin was published in Laid-open Japanese Patent Publication No.Hei8-245421; however, the method is restricted in a composition of the agent, because this method is required to use albumin other than amino acid.

As discussed above, conventional methods for stabilizing the hemoglobin are not suitable as a method for stabilizing control material and standard material, which are required for accurate measuring hemoglobin in a clinical laboratory test. Accordingly, a method for stabilizing control material and standard material containing hemoglobin, are highly desired in the fields of pharmaceuticals and clinical laboratorytests.

Summary of the Invention

As the result of a study on a stabilization of the control material and the standard material, the present inventors found that the stability of hemoglobin was improved by incorporating with sulfur containing material, particularly a compound with a SH group, thereby establishing the present invention.

That is, one of the gists of the present invention is to provide a stabilizing agent for hemoglobin, characterized by stabilizing hemoglobin in a state of solution.

The sulfur containing agent of the present invention is of compound with SH group.

Besides these, according to the present invention, said compound with SH group may be one of the compounds selected from the group consisting of sulfur containing amino acid, such as cysteine, methionine, cystine, etc., and family thereof; and sulfur containingcompounds, such as thioglycol acid, 1-thioglycelin, thiodiglycol, mercaptoethanol, glutathione, dithiothreitol and etc., and family thereof.

According to the present invention, the compound with a SH group may be cysteine and family thereof.

The present invention is characterized in that the sulfur containing compound is contained in an amount of 0.01 to 0.00001 part by weight based on 1 weight part of hemoglobin.

According to the present invention, said hemoglobin may be hemoglobin.

According to the present invention, said hemoglobin may be glycated hemoglobin.

Another aspect of the present invention is a method for stabilizing hemoglobin of the present invention.

A further aspect of the invention is a composition characterized by incorporating hemoglobin with the stabilizing agent for hemoglobin of the present invention.

Still another aspect of the present invention involves incorporating the stabilizing agent for hemoglobin in hemoglobin of the present invention.

Still yet another aspect of the invention involves use of the sulfur containing compound in the stabilizing agent of the present invention.

Detailed Description of the Present Invention

According to the present invention, hemoglobin means hemoglobin and glycated hemoglobin. An example of the glycated hemoglobin includes HbA_{Ic}. Other examples of the

hemoglobin includes HbA_{1a}, HbA_{1b}, HbF, HbA_0, HbA_2, oxyhemoglobin, carbonylhemoglobin, methohemoglobin, cyanomethohemoglobin, alkali modified hemoglobin, and the like, and other heterohemoglobin. Besides these, modified hemoglobin, such as phosphate ester derivatives of hemoglobin, hemoglobin-polyalkylene conjugates, hemoglobin-inuren conjugates, and hemoglobin-haptglobin complexes, are also included in the present invention. Further, the hemoglobin of the present invention may be available not only in human origin, but also animal origin, for example, cow, pig, sheep, horse, dog, monkey, rabbit, chicken, and the like. These are used as a standard or control material for various driedsubstances, as a lyophilized agent, and maybe in a form of dried agent, liquid agent, etc., if desired.

The first and principal aspect of a stabilizing method and stabilizing agent for hemoglobin according to the present invention, resides in insuring stability of hemoglobin in a liquid state. This means securing stability of the hemoglobin in a dried state and in a state of solution.

A stabilizing method for the hemoglobin according to the present invention is to incorporate a sulfur containing compound in order to stabilize thereof. Addition of the sulfur containing compound is more convenient by adding it in a process for preparing agent; however, such may be attained by conventionally adding after dissolving the agent, on demand. The hemoglobin standard material and control material, in which the hemoglobin is the main ingredient and the sulfur containing compound is contained as a stabilizing agent, are provided by adding the sulfur containing agent in a step for preparing agent. If the agent is to be the type, of which the compound is added on demand, the main ingredient and the stabilizing agent containing sulfur containing agent are separately prepared in the form of a kit product.

As the sulfur containing agent according to the present invention, compounds with SH group are well-known and widely available. Compound with SH groups include sulfur

containing amino acid, such as cysteine, methyonin, etc.; sulfur containing compound, such as thiobenzoic acid, thioglycol acid, 1-thioglycerine, thiodiglycol, mercaptoethanol glutathione, dithiothreitol, etc., and family thereof. These may be used singly or in combination. The most preferable is the sulfur containing amino acid, such as cysteine, methionine, cystine, and the like, and family thereof. Particularly preferred is cysteine and family thereof.

The amount of the sulfur containing agent to be used is 0.01 to 0.00001 part by weight per 1 part by weight of the hemoglobin, preferably 0.001 to 0.0001 part by weight. The concentration of a solution, when the agent is prepared in the form of a solution, is 0.01 to 100 mM, and preferably 0.1 to 10 mM, per hemoglobin of 2 to 10 weight % concentration.

The standard material or control material containing hemoglobin, which is provided and assures stability according to the present invention, may be said to be a composition containing hemoglobin. Said composition maybe incorporated applying a well-known techniques, in addition of the main ingredient and stabilizer, as base component, with bulking agents, pH control agents for protecting turbidity onsolubility thereof, protein, sucrose, high molecular weight compounds, inorganic salts, chelating agents, and the like, or may be combined thereof, on demand.

Examples thereof includes various buffer solution or pH control agent, which is capable of controlling the pH in a range of 5 to 9, proteins such as albumin, gelatin, etc., saccharides, such as glycerol, sucrose, (preferably disaccharide such as sucrose), etc., polysaccharide such as sodium sulfate dextran, heparin, sodium sulfate, chondroitin, dextran, etc., and high molecular compound such as polyethyleneglycol, polyvinyl alcohol, polyvinyl pyrroridone, polypropylene glycohol, etc. These materials maybe used singly or in combination. Besides these, there may be used saccharides such as glucose, maltose, inositol, fructose, glucitol, glucono-δ-lactone, trehalose, maltitol, raffinose, mannitol; inorganic

compound such as sodium chloride, sodium phosphate, potassium chloride, calcium lactate, etc., chelating agent such as EDTA (ethyl enediaminetetraacetic acid), NTA (nitrilotriacetic acid), EDDA (ethylenediaminediacetic acid), CyDTA (trans-1,2-Diaminocycloheane-N,N,N',N'-tetraacetic acid monohydrate), DPTA-OH (1,3-Diamino-2-hydroxyoropane N, N, N' , N' -tetraacetic acid), DTPA (Diethylenetriamine-N, N, N' ,N",N"-pentaacetic acid), EDDP (Ethylenediamine-N, N' -dipropionic acid, dihydrochloride), EDDPO [Ethylenediamine-N,N'-bis(methylenephosphosphonic acid), hemihydrate], EGTA [Ethyleneglycol-bis-((β-amino-ethylether) tetraacetic acid], HBED [N,N'-bis (2-hydroxybenzyl) ethylenediamine-N,N-diacetic acid], HDTA (1,6-Hexamethylenediamine-N, N, N',N'-tetraacetic acid), HIDA [N-(2-Hydroxyethyl) iminodiacetic acid], IDA (Iminodiacetic acid), NTP (Nitrilotripropionic acid), NTPO [Nitrilotris (methylenephosphonic acid), trisodium salt], TTHA (Triethylenetetramine-N, N, N' , N", N"', N"-hexaacetic acid) , etc., a-, 3-f y-CD (cyclodextrin), or these CD modified with polymer, and the like. These may be used singly or in combination.

Examples

The present invention is explained in more detailed with referring to the following examples, which are not considered as restricting the present invention.

(Example 1)

The mixture of, an agents stated below, were prepared with or without adding 1 mM L-cysteine, 1 mL mixture were pipetted into 5 mL glass vessel, and were lyophilized to form lyophilized preparation. As hemoglobin, hemoglobin standard containing HbA_{1a}, HbA_{1b}, HbF, and HbA_0 were used, and added in a concentration of 7%.

10 mm	Phosphate buffer solution (pH 7.0)
25 mM	EDTA•2Na
7 %	Hemoglobin standard (JML company)
25 %	Sucrose

(Experimental Example 1)

Each of the lyophilized agents, with or without incorporating L-cysteine, which were prepared in the Example 1, was dissolved in 1 mL distilled water, to compare stability of the hemoglobin in a state of solution, at 25 °C, after 25 hours elapse of time. Measurement of the hemoglobin was performed by detecting absorbance (0. D. value) at 577 nm. (Method in Enzymology, 188, 266-272) . From the results, as shown in the Table 1, by adding L-cysteine, a sufficient stabilizing effect of the hemoglobin in a state of solution was confirmed. Further, a chemical analysis was conducted of each portion using an automatic glycated hemoglobin analyzer HLC-723GHbIII, manufactured by Toso company. These results showed that stability was provided in all kinds of hemoglobin fractions.

Table 1

	0 Hour	25 Hour
With L-cysteine adding	0.86	0.85
Without L-cysteine	0.86	0.41

(Example 2)

A sample was prepared with the agent, stated below, by adding with or without adding 1 mM L-cysteine. In the same manner, as stated in the Example 1, the hemoglobin standard containing HbA_{1c}, HbA_{1a}, HbF, and HbA_0 was used in order to make a concentration of hemoglobin in 7%.

10 mm	Phosphate buffer solution (pH 7.0)
25 mM	EDTA 2 Na
7 %	Hemoglobin standard (JML company)

(Experimental Example 2)

Each of the agents, with or without incorporating L-cysteine, which were prepared in the Example 2, was studied in comparing stability of hemoglobin after preparation, and at 25°C after 25 hours elapse of time, in the same manner as of the Experimental Example 1. The results are shown in Table 2. According to adding cysteine, a sufficient stability of the hemoglobin was obtained, regardless of presence of sucrose. Further, chemical analysis of each portion was carried out using Automatic Analyzer of glycohemoglobinHLC-723GHbIII. These results showed that stability was provided in all kinds of hemoglobin fractions.

Table 2

	0 Hour	25 Hour
With L-cysteine adding	0.85	0.85
Without L-cysteine	0.85	0.43

Possibility in commercially available

By the stabilizing method for hemoglobin and stabilizing agent, characterized in adding sulfur containing compound according to the present invention, the stability of hemoglobin and glycated hemoglobin in a state of solution was secured. Thus, by introducing stabilizing means for hemoglobin, for example, the stability of a standard material and controlling material containing hemoglobin for clinical laboratory test was improved, resulting in an expecting highly accurate laboratory test results. The present invention is useful for contributing in the fields of clinical laboratory tests and pharmaceuticals.